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When do myopia genes have their effect? Comparison of genetic risks between children and adults

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When do myopia genes have their effect? Comparison of genetic risks between children and adults

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ABSTRACT

Purpose: Previous studies have identified many genetic loci for refractive error and myopia. We aimed to investigate the effect of these loci on ocular biometry as a function of age in children, adolescents and adults.

Methods: The study population consisted of three age-groups identified from the international CREAM consortium: 5,490 individuals aged <10 years; 5,000 aged 10-25 years; and 16,274 aged >25 years. All participants had undergone standard ophthalmic examination including measurements of axial length (AL) and corneal radius (CR). We examined the lead SNP at all 39 currently known genetic loci for refractive error identified from genome-wide association studies (GWAS), as well as a combined genetic risk score (GRS). The beta coefficient for association between SNP genotype or GRS versus AL/CR was compared across the 3 age groups, adjusting for age, sex, and principal components. Analyses were Bonferroni-corrected.

Results: In the age-group <10 years, 3 loci (*GJD2, CHRNG, ZIC2*) were associated with AL/CR. In the age-group 10-25 years, 4 loci (*BMP2, KCNQ5, A2BP1, CACNA1D*) were associated; and in adults 20 loci were associated. Association with GRS increased with age; β = 0.0016 per risk allele (*P* = 2E-08) in <10 years, 0.0033 (*P* = 5E-15) in 10-25 year-olds, and 0.0048 (*P* = 1E-72) in adults. Genes with strongest effects (*LAMA2*, *GJD2*) had an early effect that increased with age.

Conclusion: Our results provide insights on the age span during which myopia genes exert their effect. These insights form the basis for understanding the mechanisms underlying high and pathological myopia.

Key words: myopia, genetic risk, development, SNPs

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INTRODUCTION

The prevalence of myopia (nearsightedness) has increased dramatically in developed countries in recent decades [Bar Dayan, et al. 2005; Vitale, et al. 2009]. Myopia is a complex, multifactorial disease with increasing public health burden due to a strong rise worldwide. In particular high myopia is associated with blinding complications such as myopic macular degeneration, glaucoma and retinal detachment [Curtin and Karlin 1971; McBrien and Gentle 2003; Saw 2006]. High myopia mostly has its onset in early childhood before age 10 years [Fledelius 2000].

The eye's dimensions alter markedly during the peak development phase between birth and the late teenage years, ultimately exerting very strong effects on final refractive error (RE) in later adult life. A complex process called emmetropisation aims to coordinate ocular development, bringing light into clear focus on the retina. Early life myopia is characteristically associated with excessive axial length (AL) increase. This results in a mismatch of the optical effects of the various refractive components of the eye, resulting in a focal point in front of the retina. Such a mismatch can be described by the ratio of AL to corneal radius (CR), AL/CR ratio, which has a high correlation with RE [Hashemi, et al. 2013; Ip, et al. 2007] and is independent of cycloplegia which may vary between studies.

Various studies have examined the heritability of myopia showing increased risk for first-degree relatives of affected individuals [Farbrother, et al. 2004; Guggenheim, et al. 2000] and twins [Sanfilippo, et al. 2010; Young, et al. 2007]. Numerous genetic loci that cause familial high myopia (*MYP1-18*) have been discovered using linkage analysis [Baird, et al. 2010]. More recently, genome wide association studies (GWAS) in large cohorts have been performed to identify further determinants for REs in the general population. The first single nucleotide polymorphisms (SNPs) identified were near *GJD2* [Solouki, et al. 2010] and *RASGRF1* [Solouki, et al. 2010]. Later many more loci were found in studies of large populations (CREAM; 23andMe)[Kiefer, et al. 2013; Verhoeven, et al. 2013] [Wojciechowski and Hysi 2013].

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All previously published refractive error GWAS studies were performed in cohorts enrolling participants aged 25 years and older. We aimed to study the effect size of the 39 GWAS-identified genetic regions associated with refractive error to date, as a function of age.

METHODS

Study specific analysis

We included 18 cohorts from 8 different countries in Europe, Asia and Oceania, with a total of 5,490 children <10 years, 5,000 individuals of 10-25 years, and 16,274 adults, all with phenotypic and genome-wide genotypic data available. Details on subject recruitment procedures can be found in the supplemental materials. Each study participant was genotyped with either an Affymetrix or Illumina SNP array (supplemental table I). All studies were conducted according to the Declaration of Helsinki. The studies were approved by the local review boards. Written, informed consent for the collection and analysis of measurements of all study participants was obtained.

SNPs

A total of 39 SNPs were included in this analysis. The SNPs were selected based on their known association with RE and myopia in the GWAS carried out by CREAM [Verhoeven, et al. 2013] and 23andMe [Kiefer, et al. 2013](supplementary table II). An unweighted genetic risk score (GRS) was calculated for each participant by summing the dosage of risk alleles (scale 0-2) for all 39 SNPs. The risk score was normally distributed.

Ocular biometry

The ocular biometry measurements included AL and CR, and the AL/CR ratio was calculated. Multiple measurements of AL and CR were taken of the right eye and left eye, were averaged to calculate a mean AL and CR for each eye. The average AL of both eyes was divided by the average CR of both eyes to calculate the AL/CR ratio. Details of the phenotypic assessment protocols/instruments used in each study can be found in the supplemental material.

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Meta-analysis

All studies performed linear regression models with each SNP or the GRS as determinants, and the AL/CR ratio as outcome. Analyses were adjusted for the potentially confounding effects of age and gender, and additionally – to account for ancestry differences within the sample – for principal components where applicable. A meta-analysis was performed to estimate the beta effects using an inversed variance weighted fixed effect model with METAL [Willer, et al. 2010]. Meta-analyses were performed in each age stratum separately, and in combined strata of all participants <25 years. Several children measured in TEST (Twins Eye Study Tasmania) and GTES (Guangzhou Twin Eye Study) had follow up measurements at an older age; therefore, only data from the oldest age were used in the combined analysis. In the Asian studies the following SNPs were excluded due to low minor allele frequency (MAF) <0.05 in the Chinese population: rs17428076, rs1656404, rs14165, rs13091182, rs12205363, rs11145465, rs10882165, and rs17183295.

Pathway analysis

Loci with significant effects (*P* <0.05) were further explored to identify differences in effect of early-onset genes (significant loci identified in groups <10 years, 10-25 years or the combined analysis) and late-onset genes (adult subjects). Data were analysed through the use of QIAGEN's Ingenuity®

Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) and the online software tool Database for Annotation, Visualization and Integrated Discovery (DAVID) [Huang da, et al. 2009a; Huang da, et al. 2009b].

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RESULTS

Our study sample of children <10 years comprised 5,490 participants derived from 5 studies; one of European ancestry (TEST), three of Asian ancestry (SCORM, STARS, and Guangzhou Twins), and one of mixed European, African, and Asian ancestry (Generation R). Our sample of individuals aged 10-25 years included 5,000 participants derived from 6 studies; 4 of European ancestry (TEST, ALSPAC, BATS and RAINE) , and 2 of Asian (STARS, Guangzhou Twins) ancestry. Our sample of adults >25 years compromised 16,274 participants derived from 10 studies; 9 of European ancestry (Croatia Split, -Kurcula and – Vis study, Gothenburg Health Study, EPIC-Norfolk and the Rotterdam Study I-III), and one Asian study (Nagahama). General characteristics per study are shown in Table I.

Genetic risk score

The genetic risk score was associated with a higher AL/CR ratio even in children aged <10 years (table II), and this association increased in magnitude with older age. Specifically, AL/CR increased with each age category from β 0.0019 (SD 0.0003) per risk allele in children <10 years, to 0.0033 (SD 0.0004) in participants aged 10-25 years, to 0.0051 (SD 0.0003) in adults (figure I). Only the adult group showed evidence for heterogeneity (heterogeneity *P*-value 0.0005) between studies, therefore, meta-analyses for this age category were also performed using the random effect model (β 0.0048; SD 0.0007; supplementary table IV). The variance explained by the genetic risk score increased from 0.7% in the children aged 6 from the Generation R study, to 3.7% for the adult participants in the RS I-III (Fig II).

Genetic loci

In children <10 years, 9/39 loci were significant at P <0.05, and 3/39 were significant after correction for multiple-testing for 39 SNPs (P <0.00128). The 3 loci significant after Bonferroni correction were in the vicinity of the genes *GJD2*, *ZIC2* and *CHRNG*. The 2 nominally-significant

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loci with the greatest effect size (beta) were close to the *CHRNG* and *PRSS56* genes. The other 5 loci were near *KCNQ5, SHISA6, KCNMA1, BMP2* and *BICC1*. Interestingly, the SNP at the *BMP2* locus had a reversed effect from that observed in adult samples, i.e., the risk allele was associated with a lower AL/CR ratio. In individuals aged 10 - 25 years, 10/39 loci showed nominally significant association with AL/CR ratio, of which 5 survived Bonferroni correction (*BMP2, TOX, KCNQ5, A2BP1* and *CACNA1D).* Five of the 10 SNPs above were already nominal significantly associated with AL/CR ratio in children <10 years (*GJD2, BICC1, ZIC2, BMP2* and *PRSS56);* of the remaining nominally-significant loci, the variant with the greatest effect in 10-25 year-olds was the SNP at the *LAMA2* locus. One variant differed significantly in effect between children <10 years and those aged 10-25 years. This was the SNP at the *BMP2* locus which, as mentioned above, showed an opposite effect to that expected in children aged <10 years (Figure III). One of the loci (*TOX)* showed evidence for heterogeneity (supplementary table III) in effect between study cohorts in the age category 10-25 years (Heterogeneity *P* = 0.001). With random effect model the effect of this SNP decreased to β 0.0062 (SE 0.0073; *P* 0.40)(supplementary table IV). In the combined analysis of all studies <25 years, *BICC1* and *PRSS56* reached Bonferroni adjusted significance; one additional locus (*PDE11A)* showed a nominally significant effect for AL/CR ratio. In adults, 31/39 loci showed a significant effect, of which 19/39 were Bonferroni significant. All loci, except for *ZBTB38* (β -0.0004; SE 0.0019), showed an association in the expected direction (i.e. risk allele associated with a higher AL/CR ratio). As in 10-25 years, one locus significant in adults showed evidence for heterogeneity (LOC100506035); with random effect model this locus lost statistical significance (supplementary table III and IV). Figure IV displays all estimated effect sizes per age group.

Pathway analysis

Pathway analyses were performed to gain insight into the mechanisms for early versus late-onset eye growth and myopia development. We hypothesized that loci with at least a moderate effect in children and adolescents most likely had an early onset. Hence, a locus was defined as

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early onset when nominally significant *(P<0.05)* in the groups <25 years and no evidence for heterogeneity (Figure IV; loci above green line). Loci nominally significant in the adult population without a significant effect <25 years were grouped as late onset genes (Figure IV; loci below green line).

Ingenuity Pathway Analysis (IPA)

Genes with an early onset in the age group <25 years were enriched in pathways of auditory disease, organismal injury and abnormalities, and gastrointestinal disease (at FDR <5%). The genes that were significantly associated in adults predisposed to connective tissue disorders, developmental disorder (e.g. microphthalmia; *BMP4* and *SIX6*,), and also gastrointestinal disease (supplementary table V).

Database for Annotation, Visualization and Integrated Discovery (DAVID)

Using the categories defined above, early-onset genes were annotated to ion channels and ion transport (*CACNA1D, CHRNG, GJD2, KCNMA1* and *KCNQ5*). Late onset genes appeared to be more related to neuron differentiation and visual perception (*RORB, SIX6, RASGRF1, CHD7, RGR, RDH5* and *GRIA4*.) (supplementary table VI).

DISCUSSION:

This study identifies the age span during which the known GWAS-identified loci for refractive error have their greatest effect. The current meta-analysis suggests that specific loci had their greatest effect in young children (*CHRNG, ZIC2, KCNMA1*), while others reached the greatest effect during early teenage years (*BMP2, CACNA1D, A2BP1*). However, most appeared to have a gradual effect during the entire age span of myopia development (*LAMA2, LRRC4C, DLX1, RDH5, GRIA4, RGR, SIX6*).

Strengths of this study were the large sample size, the comparison across 3 distinct age categories, and the precision in measurements of ocular biometry. A drawback was the lack of complete cycloplegic refraction in children in several studies, which jeopardized valid

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measurements of RE in this age category. Thus, we used AL/CR ratio as an indicator of RE to avoid heterogeneity in the outcome. This ratio has a high correlation with RE [Hashemi, et al. 2013; Ip, et al. 2007] and was available from all studies in the consortium. Another limitation was the lack of power to detect statistically significant differences between the age groups for most genes. A pooled analysis would have increased statistical power, but raw data from individual participants were not available. Ideally, a study using longitudinal data of the same children over different age periods would have the best study design for the current analysis.

Little has been reported on the development and progression of myopia as a function of age; however, a number of studies investigated the relationship between development of ocular biometry related to age. Until the age of 25 years, corneal curvature, the crystalline lens, and axial length all evolve with age, and thereby influence refractive error. The cornea increases in radius until preschool age leading to flattening of the corneal curvature and decrease in refractive power [Augusteyn, et al. 2012]; the crystalline lens grows until 10 years of age, also reducing refractive power [Mutti, et al. 2012; Mutti, et al. 1998]. This decrease in refractive power is compensated by axial elongation which increases from 17 mm in newborns [Lim, et al. 2015] to 23.3 mm in 12-13 year olds [French, et al. 2012]. The average AL in emmetropic adults is 23.5 mm [Fotedar, et al. 2010; Gordon and Donzis 1985]. The highest growth rate of AL occurs in the first years of life and relates to emmetropisation; the growth rate after early teens is more gradual but mainly relates to myopisation [Gordon and Donzis 1985]. The exact age at which eye growth stops is not known; generally this occurs before age 20 years, but increase in AL has been described up to the age of 25 years in university students [Fledelius 2000; Midelfart, et al. 1992].

One of the key detected GWAS-identified loci for refractive error is on chromosome 15 near the *GJD2* gene, that encodes a gap junction protein known as CX36. This protein not only processes cone-to-cone and cone-to-rod signals [Lee, et al. 2003] but also directs signaling between other retinal cells [Feigenspan, et al. 2001; Hidaka, et al. 2004]. This cell-to-cell

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communication appears to be under regulation of light exposure and dopamine [Bloomfield and Volgyi 2009], two factors that have an established role in eye growth and myopia development. Our data suggest that *GJD2* has an early-onset, indicating that altered retinal cell signaling, perhaps via reduced light exposure and low dopamine levels, may be a first step in myopia development. As expected, some early-onset genes also had a reported role in eye development. Knockout of *LAMA2,* a gene encoding the large extracellular glycoprotein laminin-α2; causes growth retardation including smaller eyes with compressed cellular layers [Gupta, et al. 2012]. Mutations in the serine protease gene *PRSS56* cause a severe decrease of AL leading to microphthalmia [Nair, et al. 2011]. Another developmental gene is *ZIC2,* an enhancer-binding factor required for embryonic stem cell specification [Luo, et al. 2015]. This gene may be important for development of retinal architecture, as it is known to be involved in differentiation and proliferation of retinal progenitor cells [Watabe, et al. 2011], and development of retinal ganglion cell trajectories [Herrera, et al. 2003]. Strikingly, several other genes involved in eye development, such as *SIX6, CDH7*, and *DLX1*, did not show an early onset but were more significant after the age of 10 years. Other early-onset genes were ion channels such as *KCNQ5*, a potassium channel present in cone and rod photoreceptors [Zhang, et al. 2011], and *CACNA1D*, a calcium channel present in photoreceptors [Xiao, et al. 2007]. *CHRNG* has as yet an unknown role in myopia development. It encodes the γ subunit of the embryonal acetylcholine receptor, which is widely expressed in the retina [Hruska, et al. 1978; Hutchins and Hollyfield 1985], and is associated with multiple pterygium syndrome [Vogt, et al. 2012].

Several remarkable patterns of effect were notable. For instance, the lead SNPs at the *BMP2, MYO1D, PTPRR*, and *BMP4* loci showed an opposite effect in children <10 years than in those who were older. This is not uncommon in biology, as such a trajectory has also been described for the *FTO* locus in relation to body mass index in children [Sovio, et al. 2011]. Interestingly, gene expression studies of *BMP2* in chickens showed that mRNA of this gene in the retinal pigment epithelium is up- or down-regulated depending on the location of the image

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plane [Zhang, et al. 2012]. When the image was focused behind the retina, mRNA was downregulated and the vitreous chamber enlarged. This underscores a bidirectional role for *BMP2* in modulation of eye growth.

Most genes had a late onset. *BMP4* has a similar function to *BMP2* as it is also responds to optical defocus with bidirectional regulation of eye growth [Zhang, et al. 2013]. *SIX6* is a DNA-binding homeobox and has a SIX domain, which binds downstream effector molecules. It is known to influence eye size in zebrafish with knocked down *SIX6* expression [Iglesias, et al. 2014]. Other genes play a less obvious role in myopiagenesis. MYO1D is involved in membrane trafficking in the recycling pathway and expressed in oligodendrites [Benesh, et al. 2012]. *RORB,* a gene encoding a nuclear receptor-directing photoreceptor differentiation, is known to activate and generate S-opsin [Jia, et al. 2009; Srinivas, et al. 2006]. DLX1 belongs to the DLX family of homeobox transcription factors, and produces GABAergic interneurons during embryonic development.

In conclusion, our study suggests that only a small proportion of the currently known GWAS-identified loci for RE exert their full effect at a young age. Furthermore, some of the pathways previously-identified by GWAS meta-analyses [Verhoeven, et al. 2013] can now be separated into early- and late-onset pathways. For example, genes coding for ion channels typically had an early onset, while genes related to connective tissue and visual feedback mechanisms appeared to become more important at a later age. As the currently known genes play only a minor role in early-onset myopia, we question whether this type of myopia is caused by common variants in other genes, or whether rare variants with large effects determine early-onset. Future research may shed more light on genes for early-onset myopia, and unravelling these genes will open up strategies for prevention of high myopia.

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Table I Participating studies and characteristics stratified per age group

Study	N	AL/CR (SD; range)	Age (SD)	Gender, % Female
Age <10 years				
STARS	207	2.99 (0.150; 2.76 – 3.46)	5.45 (2.11)	47.3
Generation R	3,874	2.87 (0.083; 2.38 – 3.90)	6.18 (0.51)	50.3
SCORM	898	3.02 (0.112; 2.63 – 3.45)	7.48 (0.87)	47.7
TEST	166	2.94 (0.101; 2.65 – 3.25)	7.53 (1.21)	52.4
GTES	345	2.97 (0.100; 2.62 – 3.45)	8.73 (0.79)	50.1
Total	**5,490**			
Age 10-25 years				
STARS	96	3.23 (0.127; 2.95 – 3.60)	12.23 (1.7)	58.3
GTES	699	3.13 (0.147; 2.58 – 3.82)	14.83 (1.2)	52.9
TEST	182	2.99 (0.108; 2.68 – 3.51)	15.16 (4.0)	60.4
ALSPAC	1,996	2.99 (0.099; 2.57 – 3.52)	15.46 (0.3)	53.6
BATS	983	3.03 (0.106; 2.67 – 3.82)	19.07 (3.2)	53,8
RAINE	1,044	3.05 (0.104; 2.63 – 3.54)	20.04 (0.4)	48.9
Total	**5,000**			
Age >25 years				
Nagahama	2,762	3.13 (0.153; 2.62 – 3.86)	52.05 (13.8)	49.0
Croatia-Split	730	3.02 (0.128; 2.38 – 3.90)	52.16 (13.0)	61.2
Croatia Korcula	832	2.99 (0.203; 2.26 – 5.73)	56.62 (13.3)	64.7
Croatia-Vis	573	2.99 (0.121; 2.50 – 3.83)	55.93 (13.8)	60.4
GHS 2	936	3.07 (0.160; 2.50 – 4.01)	59.26 (10.6)	50.0
GHS 1	1,919	3.06 (0.151; 2.30 – 3.88)	60.17 (10.7)	47.1
EPIC-Norfolk	6,051	3.05 (0.146; 2.42 – 3.95)	68.9 (8.0)	54.3
RS I-III	2,471	3.05 (0.143; 2.43 – 3.86)	70.02 (8.8)	53.6
Total	**16,274**			

*GTES= Guangzhou Twin Eye Study, RS I-III = Rotterdam Study I-III, GHS=Gutenberg Health Study

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Table II Effect size of myopia related genes in age groups <10 years, 10-25 years, 25> years

				<10 years		10 - 25 years		Combined		>25 years	
Variant	**Chr**	**Gene**	**RA**	**Beta (SE)**	**P**	**Beta (SE)**	**P**	**Beta (SE)**	**P**	**Beta (SE)**	**P**
Allele Score	-	-	-	**0.0019 (0.0003)**	**10^-11**	**0.0033 (0.0004)**	**10^-15**	**0.0024 (0.0002)**	**10^-24**	**0.0051(0.0003)**	**10^-72**
rs1652333	1	CD55	G	0.0033 (0.0017)	0.05	0.0006 (0.0024)	0.80	0.0026 (0.0014)	0.07	**0.0084(0.0017)**	**10^-6**
rs4373767	1	ZC3H11B	T	0.0010 (0.0017)	0.55	0.0032 (0.0023)	0.16	0.0019 (0.0014)	0.16	**0.0053(0.0017)**	**0.002**
rs17412774	2	PABPCP2	A	0.0007 (0.0017)	0.69	0.0010 (0.0023)	0.67	0.0008 (0.0014)	0.57	**0.0063(0.0017)**	**10^-4**
rs17428076	2	DLX1	C	0.0017 (0.0021)	0.43	0.0029 (0.0027)	*0.28*	0.0024 (0.0017)	0.16	**0.0073(0.0021)**	**10^-4**
rs1898585	2	PDE11A	T	0.0022 (0.0019)	0.26	0.0050 (0.0029)	0.09	**0.0034 (0.0017)**	**0.04**	**0.0057(0.0021)**	**0.007**
rs1656404	2	PRSS56	A	**0.0073 (0.0024)**	**0.002**	**0.0067 (0.0033)**	**0.04**	**0.0069 (0.0019)**	**10^-4**	**0.0079(0.0024)**	**0.001**
rs1881492	2	CHRNG	T	**0.0086 (0.0024)**	**10^-4**	0.0039 (0.0031)	0.21	**0.0064 (0.0020)**	**0.001**	**0.0085(0.0022)**	**10^-5**
rs14165	3	CACNA1D	G	0.0035 (0.0020)	0.08	**0.0082 (0.0026)**	**0.001**	**0.0055 (0.0016)**	**0.001**	**0.0055(0.0020)**	**0.005**
rs13091182	3	ZBTB38	G	0.0008 (0.0020)	0.69	-0.0001 (0.0024)	0.98	0.0007 (0.0015)	0.66	-0.0004(0.0019)	0.83
rs9307551	4	LOC100506035	A	0.0007 (0.0019)	0.70	0.0037 (0.0026)	0.16	0.0020 (0.0016)	0.20	**0.0051(0.0020)**	**0.008**
rs5022942	4	BMP3	A	0.0014 (0.0018)	0.44	-0.0016 (0.0026)	0.54	0.0007 (0.0015)	0.63	0.0006(0.0020)	0.78
rs7744813	6	KCNQ5	A	**0.0050 (0.0017)**	**0.004**	**0.0081 (0.0023)**	**10^-4**	**0.0060 (0.0014)**	**10^-5**	**0.0066(0.0018)**	**10^-4**
rs12205363	6	LAMA2	T	0.0041 (0.0041)	0.31	**0.0138 (0.0046)**	**0.003**	**0.0094 (0.0031)**	**0.003**	**0.0229(0.0036)**	**10^-10**
rs7829127	8	ZMAT4	A	0.0025 (0.0020)	0.22	0.0019 (0.0028)	0.49	0.0025 (0.0017)	0.13	**0.0072(0.0021)**	**0.001**
rs7837791	8	TOX	G	0.0029 (0.0016)	0.06	**0.0083 (0.0022)**	***10^-4***	**0.0050 (0.0013)**	**10^-4**	**0.0042(0.0017)**	**0.012**
rs4237036	8	CHD7	T	0.0001 (0.0018)	0.96	0.0032 (0.0024)	0.18	0.0013 (0.0014)	0.37	**0.0058(0.0018)**	**0.001**
rs11145465	9	TJP2	A	0.0035 (0.0022)	0.11	0.0027 (0.0028)	0.33	0.0029 (0.0017)	0.09	**0.0062(0.0021)**	**0.004**
rs7042950	9	RORB	G	0.0028 (0.0019)	0.14	0.0031 (0.0026)	0.24	0.0027 (0.0016)	0.08	**0.0071(0.0020)**	**10^-4**
rs7084402	10	BICC1	G	**0.0035 (0.0016)**	**0.03**	**0.0066 (0.0023)**	**0.004**	**0.0050 (0.0013)**	**10^-4**	**0.0074(0.0017)**	**10^-6**
rs6480859	10	KCNMA1	T	**0.0040 (0.0018)**	**0.02**	0.0037 (0.0023)	0.10	**0.0040 (0.0014)**	**0.004**	0.0015(0.0017)	0.38
rs745480	10	RGR	G	0.0007 (0.0016)	0.67	0.0021 (0.0022)	0.34	0.0011 (0.0013)	0.40	**0.0055(0.0017)**	**0.001**
rs10882165	10	CYP26A1	T	0.0012 (0.0018)	0.49	0.0002 (0.0024)	0.93	0.0007 (0.0014)	0.61	0.0011(0.0018)	0.54
rs1381566	11	LRRC4C	G	0.0026 (0.0020)	0.21	0.0040 (0.0034)	0.23	0.0028 (0.0018)	0.12	**0.0093(0.0022)**	**10^-5**
rs2155413	11	DLG2	A	0.0022 (0.0017)	0.18	0.0027 (0.0022)	0.23	0.0023 (0.0013)	0.09	0.0021(0.0017)	0.21
rs11601239	11	GRIA4	C	0.0011 (0.0016)	0.50	0.0027 (0.0022)	0.22	0.0014 (0.0013)	0.30	**0.0055(0.0017)**	**0.001**
rs3138144	12	RDH5	G	0.0020 (0.0021)	0.35	0.0039 (0.0027)	0.16	0.0028 (0.0017)	0.10	**0.0045(0.0019)**	**0.02**
rs12229663	12	PTPRR	A	-0.0023 (0.0019)	0.21	0.0046 (0.0026)	0.08	0.0000 (0.0016)	1.00	**0.0069(0.0019)**	**10^-4**
rs8000973	13	ZIC2	C	**0.0058 (0.0017)**	**10^-4**	**0.0058 (0.0023)**	**0.01**	**0.0059 (0.0014)**	**10^-5**	0.0027(0.0017)	0.10
rs2184971	13	PCCA	A	0.0008 (0.0016)	0.61	0.0006 (0.0023)	0.80	0.0009 (0.0014)	0.48	0.0021(0.0017)	0.21
rs66913363	14	BMP4	G	-0.0025 (0.0017)	0.15	0.0040 (0.0024)	0.10	0.0006 (0.0014)	0.68	**0.0047(0.0017)**	**0.006**
rs1254319	14	SIX6	A	0.0007 (0.0017)	0.68	0.0044 (0.0024)	0.07	0.0017 (0.0014)	0.22	**0.0054(0.0018)**	**0.002**
rs524952	15	GJD2	A	**0.0069 (0.0016)**	**10^-5**	**0.0068 (0.0023)**	**0.003**	**0.0067 (0.0013)**	**10^-7**	**0.0122(0.0016)**	**10^-14**
rs4778879	15	RASGRF1	G	0.0018 (0.0017)	0.29	0.0033 (0.0023)	0.15	0.0019 (0.0014)	0.17	**0.0051(0.0017)**	**0.002**
rs17648524	16	A2BP1	C	0.0018 (0.0018)	0.33	**0.0079 (0.0024)**	**0.001**	**0.0039 (0.0015)**	**0.01**	**0.0077(0.0019)**	**10^-5**
rs2969180	17	SHISA6	A	**0.0035 (0.0016)**	**0.03**	0.0017 (0.0023)	0.46	**0.0027 (0.0014)**	**0.05**	**0.0079(0.0017)**	**10^-6**
rs17183295	17	MYO1D	T	-0.0033 (0.0023)	0.16	0.0009 (0.0030)	0.76	-0.0018 (0.0018)	0.33	**0.0089(0.0023)**	**10^-4**

rs4793501	17	KCNJ2	T	0.0029 (0.0016)	0.08	0.0001 (0.0022)	0.95	0.0019 (0.0013)	0.16	**0.0041(0.0017)**	**0.015**
rs12971120	18	CNDP2	A	0.0002 (0.0019)	0.93	0.0048 (0.0026)	0.07	0.0017 (0.0015)	0.27	0.0024(0.0019)	0.22
rs235770	20	BMP2	T	**-0.0043 (0.0018)**	**0.02**	**0.0121 (0.0025)**	**10^-6**	0.0008 (0.0015)	0.60	**0.0043(0.0017)**	**0.013**

Values are betas (SE) and *P*-values, from linear regression models adjusted for sex, age and principal components if applicable meta-analysed with inversed variance meta-analysis in METAL. Bold: *P* <0.05.

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Figure I. Association between genetic risk score and myopia in the three age groups

Figure II. Association between non-weighted genetic risk score and AL/CR ratio in children and adults.

Figure III. Increased effect on AL/CR ratio with age for *BMP2* gene.

Figure IV. Distribution of effects on AL/CR ratio per myopia-related gene in three age groups

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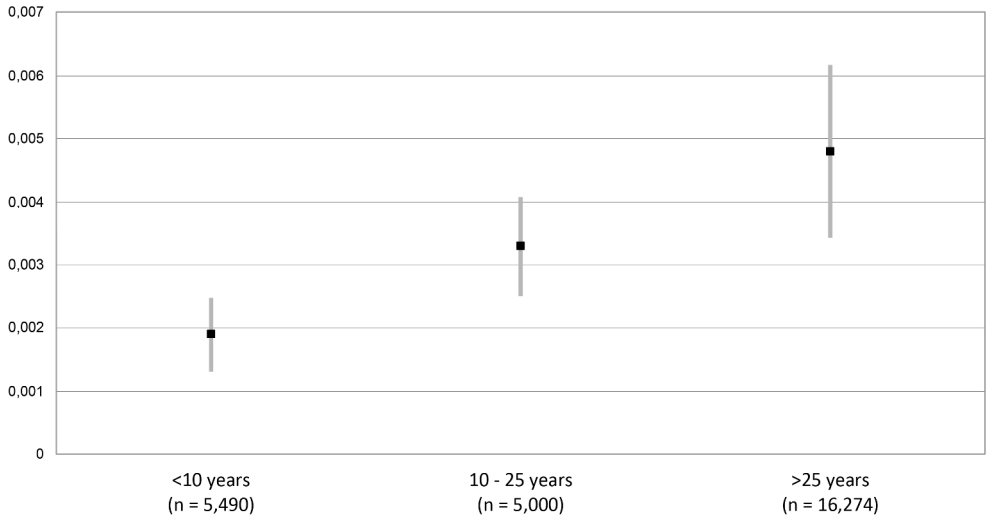

The y-axis represents the beta of the non-weighted genetic risk score. Black dots and grey lines depict the beta and the 95% CI. Estimate for > 25 years was based on a meta-analysis using a random effects model because relatively high heterogeneity; in the other two groups a fixed effects model could be used.

Figure I. Association between

258x136mm (300 x 300 DPI)

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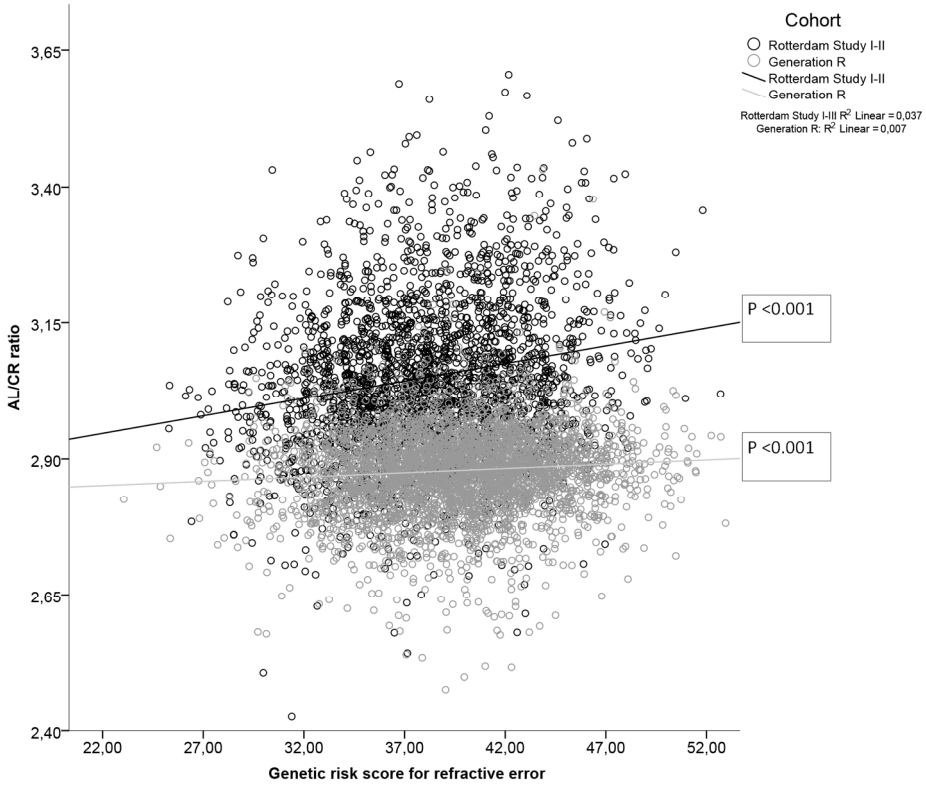
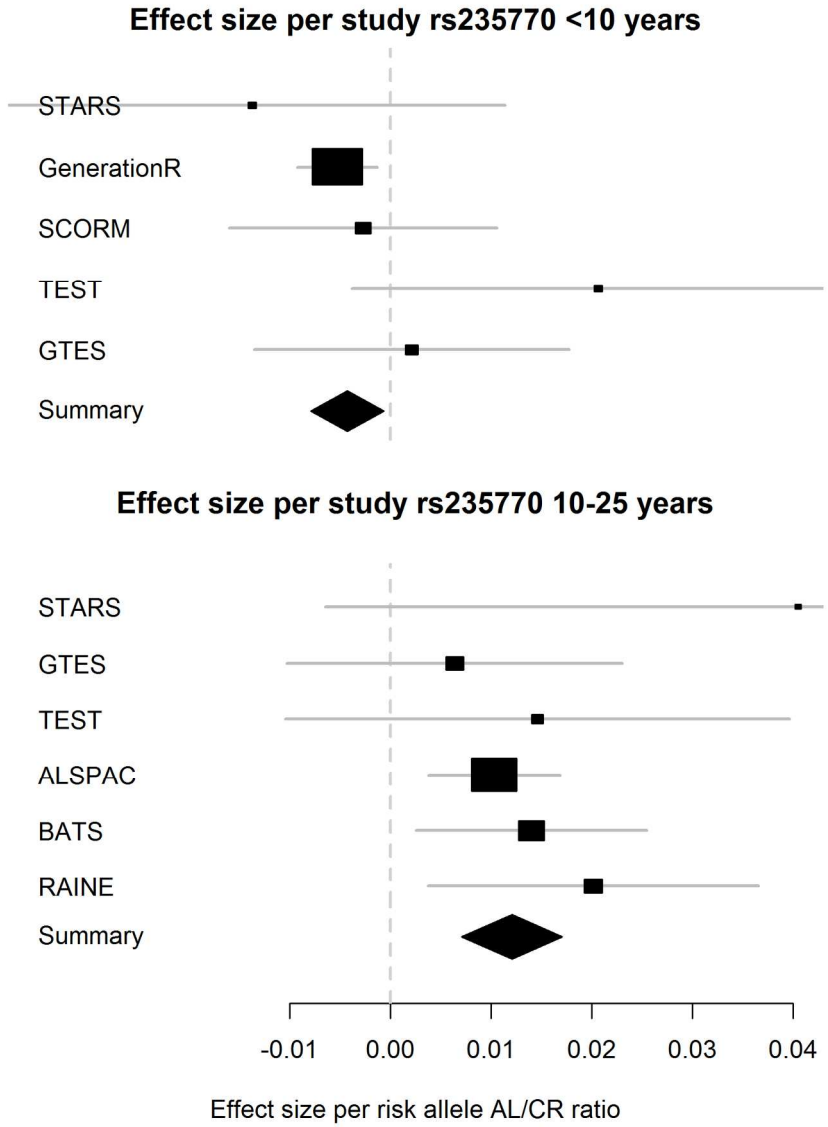


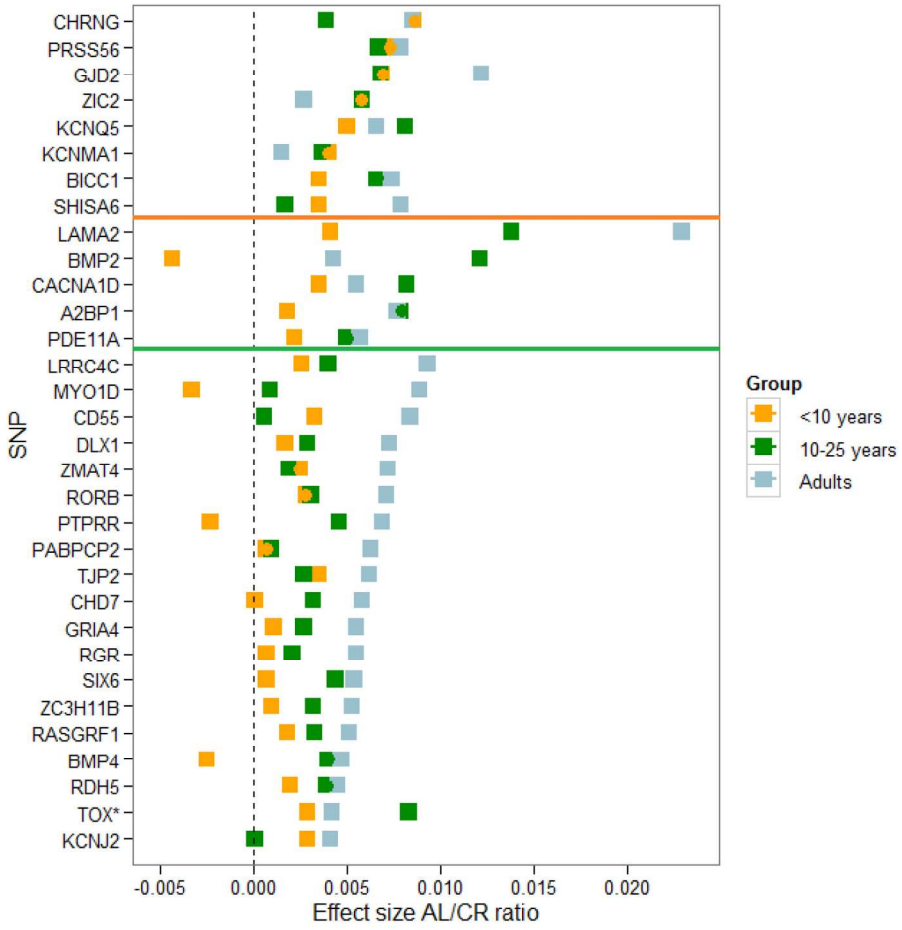
The grey dots and line represent children in Generation R (N = 3,874); the black dots and line represent adults from the Rotterdam Study I-III (N = 2,471).

Figure II. Association between

164x132mm (300 x 300 DPI)



Comparison of the association with AL/CR ratio (beta) of the topSNP near BMP2 between the age groups <10 years and 10-25 years ordered on average age for top to bottom.
Figure III. Increased effect o
169x201mm (300 x 300 DPI)



Effect was represented by betas of association with AL/CR ratio per top SNP. Above the orange line are genes that have a significant (P <0.05) effect in children <10 years; between the orange and green line are genes that have a significant effect in individuals <25 years; below the green line are genes that have a significant effect in adults. * showed heterogeneity in 10-25 years and was not significant with random effect model.

Figure IV. Distribution of eff
171x166mm (300 x 300 DPI)

When do myopia genes have their effect? Comparison of genetic risks between children and adults

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Supplements:

Table SI: Genotyping and imputation details

Table SII: SNPs previously associated with myopia and refractive error

Table SIII: Heterogeneity of the results

Table SIV: results of random effect meta-analysis in case of heterogeneity

Table SV: Results IPA pathway analysis

Table SVI: Results DAVID pathway analysis

Description of participating studies

Study specific acknowledgements

References of supplemental material

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Supplementary Table I Genotyping and imputation details

Study	Genotyping platform	Imputation	Reference population (1000G)	QC
ALSPAC	Illumina HumanHap550	MACH/minimac	GIANT phase1 release v3	Cheng et al. 2013 [1]
BATS/TEST	Illumina HumanHap610/660-Quad	MACH	1000G Phase 1 release on Aug 4, 2010	Yazar et al. 2015 [2]
RAINE	Illumina 660W-Quad	MACH/minimac	1000G Phase 1 release on Nov 23, 2010	Yazar et al. 2015 [2]
TEST	Illumina HumanHap610/660-Quad	MACH	1000G Phase 1 release on Aug 4, 2010	Yazar et al. 2015 [2]
Generation R	Illumina Infinium II HumanHap610 Quad Arrays	MACH	1000 Genomes GIANTv3 panel	Kruithof et al. 2014 [3]
GTES	Affymetrix Gene Titan	IMPUTE2 v2.3.0	1000G Phase 1 release on Nov 23,2010	
SCORM	Illumina HumanHap550/550-Duo	MACH/minimac	1000G Phase 1 release March 2012	Cheng et al. 2013 [1]
STARS	Illumina HumanHap610-Quad	MACH/minimac	1000G Phase 1 release March 2012	Cheng et al. 2013 [1]
GHS 1/2	Affymetrix Genome-Wide Human SNP Array 6.0	MACH/minimac	1000G Phase 1 release on Nov 23, 2010	
Rotterdam Study	RS I: Illumina Infinium II HumanHap550 chip v3.0 array. RS II: HumanHap550 Duo Arrays + Human610 -	MACH	NCBI build 36, HapMap release #22	Solouki et al. 2010[4]

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Croatia	Quad Arrays Illumina, RS-III: Human 610 Quad Arrays Illumina Korcula: Illumina CNV370v1 and CNV370-Quadv3 Vis: Illumina HumanHap 300v1 Split: Illumina CNV370-Quadv3 and Illumina OmniExpress Exome-8v1_A	IMPUTEv2 (phasing using shapeit v2)	1000G Phase 1 integrated v3 release March 2012 (Vis and Korcula) release June 2014 (Split)
Nagahama	Human 610 Quad Arrays Illumina / Human Omni 2.5 Arrays Illumina	MACH	NCBI build 36, HapMap release #28
EPIC-Norfolk	Affymetrix UK Biobank Axiom Array.	IMPUTE version 2.3.2.	1000G Phase 3 (October 2014)

Abbreviations: 1000G, One thousand genomes project. QC, Quality control.

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Supplementary Table II. All SNPs previously associated with myopia and refractive error.

SNP	Chr	Pos	Gene	Citation
rs1652333	1	207470460	*CD55*	Verhoeven et al. 2013
rs4373767	1	219759682	*ZC3H11B*	Cheng et al. 2013
rs17412774	2	146773948	PABPCP2	Kiefer et al. 2013
rs17428076	2	172851936	DLX1	Kiefer et al. 2013
rs1898585	2	178660450	*PDE11A*	Kiefer et al. 2013
rs1656404	2	233379941	*PRSS56*	Verhoeven et al. 2013
rs1881492	2	233406998	*CHRNG*	Verhoeven et al. 2013
rs14165	3	53847408	*CACNA1D*	Verhoeven et al. 2013
rs13091182	3	141133960	ZBTB38	Kiefer et al. 2013
rs9307551	4	80530671	*LOC100506035*	Verhoeven et al. 2013
rs5022942	4	81959966	*BMP3*	Kiefer et al. 2013
rs7744813	6	73643289	*KCNQ5*	Verhoeven et al. 2013
rs12205363	6	129834628	*LAMA2*	Verhoeven et al. 2013
rs7829127	8	40726394	*ZMAT4*	Verhoeven et al. 2013
rs7837791	8	60179086	*TOX*	Verhoeven et al. 2013
rs4237036	8	61701057	*CHD7*	Verhoeven et al. 2013
rs11145465	9	70989531	*TJP2*	Verhoeven et al. 2013
rs7042950	9	77149837	*RORB*	Verhoeven et al. 2013
rs7084402	10	60265404	*BICC1*	Verhoeven et al. 2013
rs6480859	10	79081948	KCNMA1	Kiefer et al. 2013
rs745480	10	85986554	RGR	Kiefer et al. 2013
rs10882165	10	94924324	*CYP26A1*	Verhoeven et al. 2013
rs1381566	11	40149607	LRRC4C	Kiefer et al. 2013

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rs2155413	11	84634790	DLG2	Kiefer et al. 2013
rs11601239	11	105556598	*GRIA4*	Verhoeven et al. 2013
rs3138144	12	56114768	*RDH5*	Verhoeven et al. 2013
rs12229663	12	71249996	*PTPRR*	Verhoeven et al. 2013
rs8000973	13	100691367	*ZIC2*	Verhoeven et al. 2013
rs2184971	13	100818092	*PCCA*	Verhoeven et al. 2013
rs66913363	14	54413001	BMP4	Kiefer et al. 2013
rs1254319	14	60903757	*SIX6*	Verhoeven et al. 2013
rs524952	15	35005885	*GJD2*	Verhoeven et al. 2013
rs4778879	15	79372875	*RASGRF1*	Verhoeven et al. 2013
rs17648524	16	7459683	*A2BP1*	Verhoeven et al. 2013
rs2969180	17	11407901	*SHISA6*	Verhoeven et al. 2013
rs17183295	17	31078272	*MYO1D*	Verhoeven et al. 2013
rs4793501	17	68718734	*KCNJ2*	Verhoeven et al. 2013
rs12971120	18	72174023	*CNDP2*	Verhoeven et al. 2013
rs235770	20	6761765	*BMP2*	Verhoeven et al. 2013

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Supplementary Table III. Heterogeneity per *P*-value per SNP for each age group.

				<10 years	10 - 25 years	Combined	>25 years
Variant	Ch	Gene	RA	Heterogeneity P	Heterogeneity P	Heterogeneity P	Heterogeneity P
Allele	-	-	-	0.07	0.08	**0.0002**	**0.0005**
rs1652333	1	CD55	G	0.40	0.25	0.23	0.18
rs4373767	1	ZC3H11B	T	0.18	0.69	0.29	0.38
rs1741277	2	PABPCP2	A	0.50	0.39	0.46	0.25
rs1742807	2	DLX1	C	0.26	0.02	0.05	0.70
rs1898585	2	PDE11A	T	0.40	0.86	0.76	0.77
rs1656404	2	PRSS56	A	0.45	0.15	0.25	0.53
rs1881492	2	CHRNG	T	0.69	0.34	0.45	0.95
rs14165	3	CACNA1D	G	0.48	0.70	0.51	0.26
rs1309118	3	ZBTB38	G	0.13	0.89	0.94	0.16
rs9307551	4	LOC100506035	A	0.94	0.78	0.92	**0.02**
rs5022942	4	BMP3	A	0.82	0.91	0.94	0.98
rs7744813	6	KCNQ5	A	0.31	0.66	0.53	0.65
rs1220536	6	LAMA2	T	0.12	0.07	0.06	0.54
rs7829127	8	ZMAT4	A	0.24	0.75	0.54	0.92
rs7837791	8	TOX	G	0.82	**0.001**	**0.002**	0.12
rs4237036	8	CHD7	T	0.35	0.94	0.84	0.89
rs1114546	9	TJP2	A	0.17	0.24	0.38	0.13
rs7042950	9	RORB	G	0.83	0.41	0.70	0.12
rs7084402	10	BICC1	G	0.58	0.38	0.52	0.83
rs6480859	10	KCNMA1	T	0.27	0.63	0.62	0.81
rs745480	10	RGR	G	0.38	0.88	0.68	0.10
rs1088216	10	CYP26A1	T	0.51	0.31	0.45	**0.03**
rs1381566	11	LRRC4C	G	0.40	0.60	0.49	0.78
rs2155413	11	DLG2	A	0.21	0.52	0.31	0.29
rs1160123	11	GRIA4	C	0.58	0.96	0.96	0.05
rs3138144	12	RDH5	G	0.67	0.72	0.83	0.43
rs1222966	12	PTPRR	A	0.41	0.18	0.06	0.97
rs8000973	13	ZIC2	C	0.44	0.61	0.65	**0.01**
rs2184971	13	PCCA	A	0.75	0.19	0.37	0.55
rs6691336	14	BMP4	G	0.62	0.22	0.10	0.57
rs1254319	14	SIX6	A	0.76	0.24	0.31	0.78
rs524952	15	GJD2	A	0.73	0.36	0.52	0.49
rs4778879	15	RASGRF1	G	0.15	0.99	0.79	0.30
rs1764852	16	A2BP1	C	0.14	0.52	0.07	0.72
rs2969180	17	SHISA6	A	0.59	0.24	0.30	0.23
rs1718329	17	MYO1D	T	0.47	0.99	0.83	0.37
rs4793501	17	KCNJ2	T	0.42	**0.03**	**0.03**	0.10
rs1297112	18	CNDP2	A	0.21	0.34	0.22	0.36
rs235770	20	BMP2	T	0.24	0.67	**4*E-5**	0.48

Supplementary Table IV Random effect analysis of SNPs with *P* < 0.05 and heterogeneity *P* <0.05

				10 - 25 years		>25 years	
Variant	**Chr**	**Gene**	**RA**	**Effect (SE)**	**P**	**Effect (SE)**	**P**
GRS	-	-	-	**-**	**-**	**0.0048 (0.0007)**	**<0.001**
rs9307551	4	LOC100506035	A	-	-	0.0066 (0.0034)	0.06
rs7837791	8	TOX	G	0.0062 (0.0073)	0.40	-	**-**

GRS = Genetic risk score

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Supplementary table V

IPA Analysis of diseases and disorders associated with early and late onset genes for myopia with p-values and molecules

Diseases and Disorders of early onset genes		
Name	**p-value range**	**Molecules**
Auditory Disease	1.80E-02 – 1.13E-05	2
Organismal Injury and Abnormalities	4.62E-02 – 1.13E-05	11
Gastrointestinal Disease	4.71E-02 – 5.75E-05	8
Hematological Disease	1.22E-02 – 1.18E-04	3
Metabolic disease	4.71E-02 – 1.18E-04	3
Diseases and Disorders of late onset genes		
Name	**p-value range**	**Molecules**
Connective tissue disorders	4.60E-02 – 1.14E-04	4
Developmental disorders	4.60E-02 – 1.14E-04	7
Gastrointestinal Disease	4.66E-02 – 1.14E-04	16
Skeletal and Muscular disorders	4.60E-02 – 1.14E-04	4
Cancer	4.66E-02 – 8.24E-04	16

Supplementary table VI

DAVID pathway analysis of functional annotation with early and late onset genes for myopia with p-values and molecules

Functional annotation of early onset genes		
GO Term	**p-value**	**Molecules**
Channel activity	1.8E-4	5
Passive transmembrane transporter activity	1.8E-4	5
Ion channel complex	3.2E-4	4
Ionic channel	6.7E-4	4
Cation channel activity	1.0E-3	4
Functional annotation of late onset genes		
GO Term	**p-value**	**Molecules**
Neurological system process	5.0E-4	7
Visual perception	1.0E-3	4
Sensory perception of light stimulus	1.0E-3	4
Cognition	1.1E-3	6
Vision	5.8E-3	3

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ALSPAC.

Pregnant women with an expected date of delivery between 1st April 1991 and 31st December 1992, resident in the former Avon health authority area in Southwest England, were eligible to participate in this population-based birth cohort study. 13,761 women were recruited. Ethical approval for the study was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees. Subject recruitment has been described previously [5]. Details of the phenotypes available and data access can be found at: http://www.bris.ac.uk/alspac/researchers/data-access/data-dictionary/. In brief, data collection has been via various methods including self-completion questionnaires sent to the mother, to her partner and after age 5 to the child; direct assessments and interviews in a research clinic. Ocular biometry (IOLmaster) was carried out when participants attended a research clinic visit at the target age of 15 years-old. DNA samples were available for 11,343 ALSPAC Children, prepared from either blood samples or lymphoblastoid-transformed cell lines.

BATS

The Brisbane Adolescent Twin Study is an ongoing study of adolescent and young-adult monozygotic (MZ) and dizygotic (DZ) twin pairs (2720 individuals) and their siblings (1179)[6]. Twins were initially recruited to the study from primary and secondary schools in South East Queensland in 1992, with new twins added at various intervals. In addition, a small number of twins have been recruited through word of mouth, or through the Australian Twin Registry. The study was approved by the human research ethics committee at the QIMR Berghofer Medical Research Institute. Twins have undergone a variety of phenotypic assessments. A 40-ml blood sample is collected from participants and parents at the initial assessment. A subset of participants also completed an extensive eye examination as part of the Twins Eye Study in Tasmania. Axial length was measured using IOLmaster, and corneal curvature was measured

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using a commercial automatic refractor/keratometer (Humphrey-598 Automatic Refractor/Keratometer; Carl Zeiss Meditec, Inc., Miami, FL).

GTES

The Guangzhou Twin Eye Study was launched in 2006, and it has completed eight consecutive annual follow-up examinations, with more than 1200 twin pairs participating. In brief, twins born in Guangzhou aged 7 to 15 years received annual eye examinations, including cycloplegic refraction, from 2006 onwards. Those with manifest strabismus, amblyopia, nystagmus, post-refractive surgery, or any ocular disease causing best-corrected visual acuity less than 20/25 were excluded from the current analysis. The study was conducted in accordance with the tenets of the World Medical Association's Declaration of Helsinki and was approved by the Ethics Review Board of the Zhongshan Ophthalmic Center of Sun Yat-Sen University. Written informed consent was obtained from the parents or legal guardians of the participants. Axial length was measured using the partial coherence interferometry (Zeiss IOLMaster, Jena, Germany). Corneal radius was performed under cycloplegia using an auto-refractor (Topcon KR8800, Tokyo, Japan).

Generation R

Generation R Study, a population-based prospective cohort study of pregnant women and their children in Rotterdam, The Netherlands. A total of 9,778 pregnant women were included in the study. All children were born between April 2002 and January 2006 [7, 8]. The children were invited at age 5 years with their mothers for examination on the research center by trained nurses. Of the 9,778 included pregnant woman 6,690 participated with their children for physical examination in the research centre at 5 years of age. The study protocol was approved by the Medical Ethical Committee of the Erasmus Medical Centre, Rotterdam (MEC 217.595/2002/20). Written informed consent was obtained from all participants. Ocular biometry (AL, corneal

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curvature (CC) was obtained with a Zeiss IOL-master. Data were collected from right and left eyes. Five measurements of axial length were taken of OD and OS and averaged. OD and OS measurements were combined to calculate a mean average axial length. Three measurement of K1 and K2 were taken of OD and OS, and were averaged. AL/CC ratio was calculated by dividing AL(mm) by CC (mm).

RAINE

The Western Australian Birth Cohort (Raine) Study is one of the largest ongoing prospective cohort studies. It was established in 1989 by recruiting around 2900 pregnant women at 16-18 weeks of gestation in Perth. The original aim of the study was to investigate how events during pregnancy and at birth influence the health and wellbeing of the newborns. This cohort has gone on to be examined every 2 years by different research groups. At the 20 year follow-up of the Raine Cohort were invited to participate in the Raine Eye Health Study (REHS) and undertake a comprehensive eye examination. This study was approved by the Human Research Ethics Committee at the University of Western Australia. During eye examination, post-cycloplegic autorefraction was performed in 1344 participant using the Nidek ARK-510A (NIDEK Co.Ltd, Tokyo, Japan) autorefractor. Ocular biometric parameters including axial length (AL) and corneal curvature were measured with IOLMaster V.5 (Carl Zeiss Meditec AG, Jena, Germany). For AL, five consecutive measurements were taken until the following criteria were satisfied: measurements within ± 0.02mm of each other, good waveform – no double peaks, acceptable signal-to-noise ratio >2.0. Any measurement outside the mentioned criteria deleted and repeated. During keratometry, three measurements within 0.3D within each meridian with careful alignment and focus were recorded.

SCORM

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This study is a school-based population study performed in Singapore. A total of 1,979 children in grades 1, 2, and 3 from three schools were recruited from 1999 to 2001 with detailed information described elsewhere[9]. The children were examined on the school premises annually by a team of eye care professionals. The GWAS was conducted in a subset of Chinese children of 1,116 subjects[10]. The phenotype used in the cross-sectional study was based on the SE measured on the 4th annual examination of the study (children at age 10 to 12 years). Complete post-filtering data on measurements and SNP data were available in 994 SCORM children.

STARS

STARS is a population-based survey of Chinese families with children residing in the south-western and western region of Singapore. Disproportionate random sampling by 6-month age groups resulted in the recruitment and subsequent eye examination of 3,009 Chinese children between May 2006 and November 2008. Details of the study design and methodology have been previously described. [11] A total of 1,451 samples from 440 nuclear families underwent eye examinations and were included for genome-wide genotyping. In all, 407 children with SE measurement had complete post-filtered genotype data.

TEST

Commencing in the late 2000, 1372 participants were recruited to the Twins Eye Study Tasmania through various methods including piggy-backing existing studies where twins had been recruited, utilizing the national twin registry, word-of-mouth and local media publicity and directly approaching schools [12]. Ethical approval was obtained from the Royal Victorian Eye and Ear Hospital, the University of Tasmania, the Australian Twin Registry (ATR). Axial length was measured using IOLmaster, and corneal curvature was measured using a commercial automatic refractor/keratometer (Humphrey-598 Automatic Refractor/Keratometer; Carl Zeiss Meditec, Inc., Miami, FL). In children, buccal swabs or Oragene saliva samples were collected.

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In adolescents, or when repeat examination was conducted several years later, a blood sample was taken and those participants who were now adults signed their own consent.

Rotterdam Study I-III

The Rotterdam Study is a prospective population-based cohort study in the elderly living in Ommoord, a suburb of Rotterdam, the Netherlands. Details of the study are described elsewhere 30. In brief, the Rotterdam Study consists of 3 independent cohorts: RSI, RSII, and RSIII. For the current analysis, 5,328 residents aged 55 years and older were included from RS1, 2,009 participants aged 55 and older from RS II, and 1,970 aged 45 and older from RS III. 99% of subjects were of European ancestry. Participants underwent multiple physical examinations with regular intervals from 1991 to present . In the fourth visit the examination included AL measurement with Lensstar [LS 900]. AL was an average of five measurements of OD and OS. CC was an average of three K1 and K2 measurement of OD and OS. The AL/CC ratio was calculated by dividing the mean average AL by the mean average CC. Exclusion criteria were bilateral cataract surgery, intra ocular procedures which influence corneal curvature or corneal refractive procedures.All measurements in RS-I-III were conducted after the Medical Ethics Committee of the Erasmus University had approved the study protocols and all participants had given a written informed consent in accordance with the Declaration of Helsinki. DNA was extracted from blood leucocytes according to standard procedures. Genotyping of SNPs was performed using the Illumina Infinium II HumanHap550 chip v3.0 array (RS-I); the HumanHap550 Duo Arrays and the Illumina Human610-Quad Arrays (RS-II), and the Human 610 Quad Arrays Illumina (RS-III). Samples with low call rate (0.336), or with sex-mismatch were excluded, as were outliers identified by the identity-by-state clustering analysis (outliers were defined as being >3 s.d. from population mean or having identity-by-state probabilities >97%). We used genomic control to obtain optimal and unbiased results and applied the inverse variance method of each effect size estimated for both autosomal SNPs that

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were genotyped and imputed in both cohorts. A set of genotyped input SNPs with call rate >98%, with minor allele frequency >0.01, and with Hardy-Weinberg P value >10−6was used for imputation. We used the Markov Chain Haplotyping (MACH) package version 1.0.15 software (Rotterdam, The Netherlands; imputed to plus strand of NCBI build 36, HapMap release #22) for the analyses. For each imputed SNP, a reliability of imputation was estimated as the ratio of the empirically observed dosage variance to the expected binomial dosage variance (O/E ratio). GWAS analyses were performed using GRIMP.

EPIC-Norfolk Eye Study

The European Prospective Investigation into Cancer (EPIC) study is a pan-European prospective cohort study designed to investigate the aetiology of major chronic diseases.1 EPIC-Norfolk , one of the UK arms of EPIC, recruited and examined 25,639 participants between 1993 and 1997 for the baseline examination.2 Recruitment was via general practices in the city of Norwich and the surrounding small towns and rural areas, and methods have been described in detail previously.3 Since virtually all residents in the UK are registered with a general practitioner through the National Health Service, general practice lists serve as population registers. Ophthalmic assessment formed part of the third health examination and this has been termed the EPIC-Norfolk Eye Study.4 In total, 8,623 participants were seen for the Eye Study, between 2004 and 2011. The EPIC-Norfolk Eye Study was carried out following the principles of the Declaration of Helsinki and the Research Governance Framework for Health and Social Care. The study was approved by the Norfolk Local Research Ethics Committee (05/Q0101/191) and East Norfolk & Waveney NHS Research Governance Committee (2005EC07L). All participants gave written, informed consent.

Refractive error was measured using a Humphrey Auto-Refractor 500 (Humphrey Instruments, San Leandro, California, USA). Biometry was conducted using non- contact partial coherence

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interferometry (IOLMaster V.4, Carl Zeiss Meditech Ltd, Welwyn Garden City, UK). For each eye, five measurements of axial length and three measurements of corneal curvature were taken. Axial length measurements were repeated if flagged as more than 0.1mm different to the others. AL/CR was calculated as described in the primary methods.

Genotyping was undertaken using the Affymetrix UK Biobank Axiom Array. SNP exclusion criteria included: call rate < 95%, abnormal cluster pattern on visual inspection, plate batch effect evident by significant variation in minor allele frequency, and/or Hardy-Weinberg equilibrium P < 10-7. Sample exclusion criteria included: DishQC < 0.82 (poor fluorescence signal contrast), sex discordance, sample call rate < 97%, heterozygosity outliers (calculated separately for SNPs with minor allele frequency >1% and <1%), rare allele count outlier, and impossible identity-by-descent values. Following these exclusions, there were no ethnic outliers. Data were pre-phased using SHAPEIT version 2 and imputed to the Phase 3 build of the 1000 Genomes project (October 2014) using IMPUTE version 2.3.2.

In total, 6051 participants had complete data for both genotypes and phenotypes; their mean age was 69 years and 54% were women.

Gutenberg Health Study (GHS 1, GHS 2)

The Gutenberg Health Study (GHS) is a population-based, prospective, observational cohort study in the Rhine-Main Region in midwestern Germany with a total of 15,010 participants at baseline and follow-up after five years. The study sample was recruited from subjects aged between 35 and 74 years at baseline exam. Exclusion criteria were insufficient knowledge of the German language to understand explanations and instructions, and physical or psychic inability to participate in the examinations in the study center. The interdisciplinary study design comprises an ophthalmological examination, general and especially cardiovascular examinations, psychosomatic evaluation, laboratory tests, and biobanking for proteomic and

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genetic analyses. The study was approved by the Medical Ethics Committee of the University Medical Center Mainz and by the local and federal data safety commissioners. According to the tenets of the Declaration of Helsinki, written informed consent was obtained from all participants prior to entering the study.

In the first follow-up, the examination included biometry measurement with the Lenstar® LS 900 (Haag Streit, Wedel, Germany). Axial length (AL) was an average of three measurements of OD and OS. Corneal curvature (CC) was an average of three K1 and K2 measurement of OD and OS. The AL/CC ratio was calculated by dividing the mean average AL by the mean average CC.

Within GHS, DNA was extracted from buffy-coats from EDTA blood samples. Genetic analysis was conducted in the first 5,000 study participants. For these, 3,463 individuals were genotyped in 2008 (GHS 1) and further 1,439 individuals in 2009 (GHS 2). Genotyping was performed for GHS 1 and GHS 2 using the Affymetrix Genome-Wide Human SNP Array 6.0 (http://www.affymetrix.com), as described by the Affymetrix user manual. Genotypes were called using the Affymetrix Birdseed-V2 calling algorithm. Individuals with low genotyping call rate, a too high level of heterozygosity (hetFDR>0.01)), with sex-mismatches, and with Non-European ancestry were excluded. After applying standard quality criteria (minor allele frequency >1%, genotype call rate >98% and P-value of deviation from Hardy-Weinberg equilibrium of >0.0001), 689,634 SNPs in 2996 individuals from GHS1 and 701,418 SNPs in 1,179 individuals from GHS2 remained for analysis (total 4175). Imputation of missing genotypes was performed using the software MACH (v1.0.18.c) and minimac (release 2012-03-14) with the reference panel 1000G Phase I Integrated Release Version 2 Haplotypes (2010-11-23 data freeze, 2012-02-14 haplotypes) for each cohort separately. Linear regression analyses were performed using ProbABEL (v0.4.1) with age and sex included in the model as covariates.

CROATIA-Korcula and CROATIA-Vis island Studies

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The CROATIA-Korčula and CROATIA-Vis studies performed on Croatian islands, are population-based, cross-sectional studies in which adult subjects were recruited for genetic studies of many medically-relevant traits including ocular biometrical traits (Vitart et al-IOVS 51,737-743). The studies received approval from relevant ethics committees in Scotland and Croatia and followed the tenets of the Declaration of Helsinki. Keratometry (CC) was measured on each eye using a NIDEK Ark30 hand-held autorefractometer/keratometer. Axial length (AL) was measured together with other biometric measures using a NIDEK A-scan device (Echoscan US-1800). Measures on eyes with a history of trauma, intra-ocular surgery, LASIK operations were removed. Genotypes were determined using the Illumina BeadStudio software. Samples with a call rate below 97 % , potentially mixed samples with excess autosomal heterozygosity or gender discrepancy (based on the sex chromosomes genotypes), and ethnic outliers (based on principal components analysis of genotypic data), were excluded from the analysis using the quality control algorithm implemented in the R package GenABEL. After exclusion of SNP with MAF < 0.01, call rate < 98% and HWE deviation p< 10-6, samples were pre-phased using shapeit v2(ref O. Delaneau, JF. Zagury, J. Marchini (2013). Improved whole chromosome phasing for disease and population genetic studies. Nat Methods. 10(1):5-6. doi: 10.1038/nmeth.2307). Imputation was carried out using impute v2 (ref B. N. Howie, P. Donnelly, and J. Marchini (2009) A flexible and accurate genotype imputation method for the next generation of genome-wide association studies. PLoS Genetics 5(6): e1000529) and the 1,000 genomes All ancestries phase1 integrated v3 reference panel. The impute2mach GENABEL function was used to convert the impute2 outputs to the MACH format that is used in the ABEL suite (http://www.genabel.org/packages) and mixed model analyses were run using the polygenic functions of the GenABEL package to account for relatedness between individuals and fitting independent SNP doses or genetic score as fixed effect together with gender.

CROATIA-Split Study

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The CROATIA-Split study, Croatia, is a population-based, cross-sectional study in the Dalmatian City of Split that includes 1000 examinees aged 18-95. The study received approval from relevant ethics committees in Scotland and Croatia and followed the tenets of the Declaration of Helsinki. Keratometry and A-scan were performed as described for the other CROATIA studies.

Individuals were genotyped with either the 370CNV-Quadv3 (n=500) or the Illumina OmniExpress Exome-8v1_A beadchips (n=500). Alleles were called in BeadStudio/GenomeStudio using Illumina cluster files. Subjects were excluded if they fulfilled any of the following criteria: genotypic call rate <97%, mismatch between reported and genotypic sex, unexpectedly low genomic sharing with first degree relatives, excess autosomal heterozygosity, or outliers identified by IBS clustering analysis. We excluded SNPs on the basis of minor allele frequency (<0.01/monomorphism), HWE (P<10^-6), call rate (<97%). The samples genotyped with the denser array (Illumina OmniExpress Exome) were first prephased and imputed as described for the CROATIA island studies; the phased data was also used as a secondary reference panel to complement the 1,000 genomes All ancestries phase1 integrated v3 reference panel for the imputation of the samples genotyped on the less dense array. Doses derived from imputations for the two halves of the study were then combined for analysis in mixed model analyses using the polygenic functions of the GenABEL package to account for relatedness between individuals and fitting independent SNP doses or genetic score as fixed effects together with gender.

Nagahama

The Nagahama Prospective Genome Cohort for the Comprehensive Human Bioscience dataset (The Nagahama Study, n=9,809) is a community-based prospective multiomics cohort study recruited from the general population living in Nagahma City. The institutional review board and

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ethics committee of Kyoto University Graduate School and the Faculty of Medicine Ethics Committee, the Ad Hoc Review Board of the Nagahama Cohort Project, and the Nagahama Municipal Review Board of Personal Information Protection approved the protocols of this study. As part of the eye examination, all participants underwent automatic objective refractometry and corneal curvature calculation (Autorefractor ARK-530; Nidek, Tokyo, Japan) and axial length (AL) measurement (IOL Master; Carl Zeiss, Jena, Germany). The AL/CC ratio was calculated by dividing the mean average AL by the mean average CC. DNA was extracted from blood leucocytes and genotyping of SNPs was performed for 3,712 samples using at least one of the three genotyping platforms, HumanHap610K Quad Arrays, HumanOmni2.5M Arrays, or HumanExome Arrays (Illumina, Inc., San Diego, CA, USA).

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When do myopia genes have their effect? Comparison of genetic risks between children and adults

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ABSTRACT

Purpose: Previous studies have identified many genetic loci for refractive error and myopia. We aimed to investigate the effect of these loci on ocular biometry as a function of age in children, adolescents and adults.

Methods: The study population consisted of three age-groups identified from the international CREAM consortium: 5,490 individuals aged <10 years; 5,000 aged 10-25 years; and 16,274 aged >25 years. All participants had undergone standard ophthalmic examination including measurements of axial length (AL) and corneal radius (CR). We examined the lead SNP at all 39 currently known genetic loci for refractive error identified from genome-wide association studies (GWAS), as well as a combined genetic risk score (GRS). The beta coefficient for association between SNP genotype or GRS versus AL/CR was compared across the 3 age groups, adjusting for age, sex, and principal components. Analyses were Bonferroni-corrected.

Results: In the age-group <10 years, 3 loci (*GJD2, CHRNG, ZIC2*) were associated with AL/CR. In the age-group 10-25 years, 4 loci (*BMP2, KCNQ5, A2BP1, CACNA1D*) were associated; and in adults 20 loci were associated. Association with GRS increased with age; β = 0.0016 per risk allele (*P* = 2E-08) in <10 years, 0.0033 (*P* = 5E-15) in 10-25 year-olds, and 0.0048 (*P* = 1E-72) in adults. Genes with strongest effects (*LAMA2*, *GJD2*) had an early effect that increased with age.

Conclusion: Our results provide insights on the age span during which myopia genes exert their effect. These insights form the basis for understanding the mechanisms underlying high and pathological myopia.

Key words: myopia, genetic risk, development, SNPs

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INTRODUCTION

The prevalence of myopia (nearsightedness) has increased dramatically in developed countries in recent decades [Bar Dayan, et al. 2005; Vitale, et al. 2009]. Myopia is a complex, multifactorial disease with increasing public health burden due to a strong rise worldwide. In particular high myopia is associated with blinding complications such as myopic macular degeneration, glaucoma and retinal detachment [Curtin and Karlin 1971; McBrien and Gentle 2003; Saw 2006]. High myopia mostly has its onset in early childhood before age 10 years [Fledelius 2000].

The eye's dimensions alter markedly during the peak development phase between birth and the late teenage years, ultimately exerting very strong effects on final refractive error (RE) in later adult life. A complex process called emmetropisation aims to coordinate ocular development, bringing light into clear focus on the retina. Early life myopia is characteristically associated with excessive axial length (AL) increase. This results in a mismatch of the optical effects of the various refractive components of the eye, resulting in a focal point in front of the retina. Such a mismatch can be described by the ratio of AL to corneal radius (CR), AL/CR ratio, which has a high correlation with RE [Hashemi, et al. 2013; Ip, et al. 2007] and is independent of cycloplegia which may vary between studies.

Various studies have examined the heritability of myopia showing increased risk for first-degree relatives of affected individuals [Farbrother, et al. 2004; Guggenheim, et al. 2000] and twins [Sanfilippo, et al. 2010; Young, et al. 2007]. Numerous genetic loci that cause familial high myopia (*MYP1-18*) have been discovered using linkage analysis [Baird, et al. 2010]. More recently, genome wide association studies (GWAS) in large cohorts have been performed to identify further determinants for REs in the general population. The first single nucleotide polymorphisms (SNPs) identified were near *GJD2* [Solouki, et al. 2010] and *RASGRF1* [Solouki, et al. 2010]. Later many more loci were found in studies of large populations (CREAM; 23andMe)[Kiefer, et al. 2013; Verhoeven, et al. 2013] [Wojciechowski and Hysi 2013].

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All previously published refractive error GWAS studies were performed in cohorts enrolling participants aged 25 years and older. We aimed to study the effect size of the 39 GWAS-identified genetic regions associated with refractive error to date, as a function of age.

METHODS

Study specific analysis

We included 18 cohorts from 8 different countries in Europe, Asia and Oceania, with a total of 5,490 children <10 years, 5,000 individuals of 10-25 years, and 16,274 adults, all with phenotypic and genome-wide genotypic data available. Age cut off points were based on prior knowledge regarding eye growth. The eye has the highest growth rate before the age of 10 years, and generally does not grow in axial length after age 25 years [Zadnik, et al. 2003]. Details on subject recruitment procedures can be found in the supplemental materials. Each study participant was genotyped with either an Affymetrix or Illumina SNP array (supplemental table I). All studies were conducted according to the Declaration of Helsinki. The studies were approved by the local review boards. Written, informed consent for the collection and analysis of measurements of all study participants was obtained.

SNPs

A total of 39 SNPs were included in this analysis. The SNPs were selected based on their known association with RE and myopia in the GWAS carried out by CREAM [Verhoeven, et al. 2013] and 23andMe [Kiefer, et al. 2013](supplementary table II). An unweighted genetic risk score (GRS) was calculated for each participant by summing the dosage of risk alleles (scale 0-2) for all 39 SNPs. The risk score was normally distributed.

Ocular biometry

The ocular biometry measurements included AL and CR, and the AL/CR ratio was calculated. Multiple measurements of AL and CR were taken of the right eye and left eye, were averaged to calculate a mean AL and CR for each eye. The average AL of both eyes was divided by the

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average CR of both eyes to calculate the AL/CR ratio. Details of the phenotypic assessment protocols/instruments used in each study can be found in the supplemental material.

Meta-analysis

All studies performed linear regression models with each SNP or the GRS as determinants, and the AL/CR ratio as outcome. Analyses were adjusted for the potentially confounding effects of age and gender, and additionally – to account for ancestry differences within the sample – for principal components where applicable. A meta-analysis was performed to estimate the beta effects using an inversed variance weighted fixed effect model with METAL [Willer, et al. 2010]. Meta-analyses were performed in each age stratum separately, and in combined strata of all participants <25 years. Several children measured in TEST (Twins Eye Study Tasmania) and GTES (Guangzhou Twin Eye Study) had follow up measurements at an older age; therefore, only data from the oldest age were used in the combined analysis. In the Asian studies the following SNPs were excluded due to low minor allele frequency (MAF) <0.05 in the Chinese population: rs17428076, rs1656404, rs14165, rs13091182, rs12205363, rs11145465, rs10882165, and rs17183295.

Pathway analysis

Loci with significant effects (P <0.05) were further explored to identify differences in effect of early-onset genes (significant loci identified in groups <10 years, 10-25 years or the combined analysis) and late-onset genes (adult subjects). Data were analysed through the use of QIAGEN's Ingenuity®.

Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) and the online software tool Database for Annotation, Visualization and Integrated Discovery (DAVID) [Huang da, et al. 2009a; Huang da, et al. 2009b].

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RESULTS

Our study sample of children <10 years comprised 5,490 participants derived from 5 studies; one of European ancestry (TEST), three of Asian ancestry (SCORM, STARS, and Guangzhou Twins), and one of mixed European, African, and Asian ancestry (Generation R). Our sample of individuals aged 10-25 years included 5,000 participants derived from 6 studies; 4 of European ancestry (TEST, ALSPAC, BATS and RAINE) , and 2 of Asian (STARS, Guangzhou Twins) ancestry. Our sample of adults >25 years compromised 16,274 participants derived from 10 studies; 9 of European ancestry (Croatia Split, -Kurcula and – Vis study, Gothenburg Health Study, EPIC-Norfolk and the Rotterdam Study I-III), and one Asian study (Nagahama). General characteristics per study are shown in Table I.

Genetic risk score

The genetic risk score was associated with a higher AL/CR ratio even in children aged <10 years (table II), and this association increased in magnitude with older age. Specifically, AL/CR increased with each age category from β 0.0019 (SD 0.0003) per risk allele in children <10 years, to 0.0033 (SD 0.0004) in participants aged 10-25 years, to 0.0051 (SD 0.0003) in adults (figure I). Only the adult group showed evidence for heterogeneity (heterogeneity *P*-value 0.0005) between studies, therefore, meta-analyses for this age category were also performed using the random effect model (β 0.0048; SD 0.0007; supplementary table IV). The variance explained by the genetic risk score increased from 0.7% in the children aged 6 from the Generation R study, to 3.7% for the adult participants in the RS I-III (Fig II).

Genetic loci

In children <10 years, 9/39 loci were significant at P <0.05, and 3/39 were significant after correction for multiple-testing for 39 SNPs (P <0.00128). The 3 loci significant after Bonferroni correction were in the vicinity of the genes *GJD2*, *ZIC2* and *CHRNG*. The 2 nominally-significant

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loci with the greatest effect size (beta) were close to the *CHRNG* and *PRSS56* genes. The other 5 loci were near *KCNQ5, SHISA6, KCNMA1, BMP2* and *BICC1*. Interestingly, the SNP at the *BMP2* locus had a reversed effect from that observed in adult samples, i.e., the risk allele was associated with a lower AL/CR ratio. In individuals aged 10 - 25 years, 10/39 loci showed nominally significant association with AL/CR ratio, of which 5 survived Bonferroni correction (*BMP2, TOX, KCNQ5, A2BP1* and *CACNA1D).* Five of the 10 SNPs above were already nominal significantly associated with AL/CR ratio in children <10 years (*GJD2, BICC1, ZIC2, BMP2* and *PRSS56);* of the remaining nominally-significant loci, the variant with the greatest effect in 10-25 year-olds was the SNP at the *LAMA2* locus. One variant differed significantly in effect between children <10 years and those aged 10-25 years. This was the SNP at the *BMP2* locus which, as mentioned above, showed an opposite effect to that expected in children aged <10 years (Figure III). One of the loci (*TOX)* showed evidence for heterogeneity (supplementary table III) in effect between study cohorts in the age category 10-25 years (Heterogeneity *P* = 0.001). With random effect model the effect of this SNP decreased to β 0.0062 (SE 0.0073; *P* 0.40)(supplementary table IV). In the combined analysis of all studies <25 years, *BICC1* and *PRSS56* reached Bonferroni adjusted significance; one additional locus (*PDE11A)* showed a nominally significant effect for AL/CR ratio. In adults, 31/39 loci showed a significant effect, of which 19/39 were Bonferroni significant. All loci, except for *ZBTB38* (β -0.0004; SE 0.0019), showed an association in the expected direction (i.e. risk allele associated with a higher AL/CR ratio). As in 10-25 years, one locus significant in adults showed evidence for heterogeneity (LOC100506035); with random effect model this locus lost statistical significance (supplementary table III and IV). Figure IV displays all estimated effect sizes per age group.

Pathway analysis

Pathway analyses were performed to gain insight into the mechanisms for early versus late-onset eye growth and myopia development. We hypothesized that loci with at least a moderate (nominally significant *P*<0.05) effect in children and adolescents most likely had an early onset.

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Hence, a locus was defined as early onset when nominally significant *(P<0.05)* in the group<10 years of age or the group 10-25 years and no evidence for heterogeneity (in Figure IV all loci above the green line). Loci nominally significant in the adult population without a significant effect in the group<10 years of age or the group 10-25 years were grouped as late onset genes (in Figure IV all loci below the green line). We utilized two types of pathway analysis software.

Ingenuity Pathway Analysis (IPA)

IPA is a web-based software to analyse and integrate the identified SNPs based on biological functions. Analysis were performed in two separate analysis, one analysis with genes with an early onset and one analysis with late onset genes. We used the program's diseases and disorder table to identify associated diseases. Genes with an early onset in the age groups <25 years were enriched in pathways of auditory disease, organismal injury and abnormalities, and gastrointestinal disease (at FDR <5%). The genes that were significantly associated in adults predisposed to connective tissue disorders, developmental disorder (e.g. microphthalmia; with the genes *BMP4* and *SIX6*), and also gastrointestinal disease (supplementary table V).

Database for Annotation, Visualization and Integrated Discovery (DAVID)

The software program DAVID is an online knowledge database to identify overlapping functions of genes. We performed the analyses separately for early and late onset genes. Using the categories defined above, early-onset genes were significantly more than expected annotated to ion channels and ion transport. The genes annotated to these categories were *CACNA1D, CHRNG, GJD2, KCNMA1* and *KCNQ5*. Late onset genes appeared to be significantly more related to neuron differentiation and visual perception. The genes involved in these categories were *RORB, SIX6, RASGRF1, CHD7, RGR, RDH5* and *GRIA4*. (supplementary table VI).

DISCUSSION:

This study identifies the age span during which the known GWAS-identified loci for refractive error have their greatest effect. The current meta-analysis suggests that specific loci had their

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greatest effect in young children (*CHRNG, ZIC2, KCNMA1*), while others reached the greatest effect during early teenage years (*BMP2, CACNA1D, A2BP1*). However, most appeared to have a gradual effect during the entire age span of myopia development (*LAMA2, LRRC4C, DLX1, RDH5, GRIA4, RGR, SIX6*).

Strengths of this study were the large sample size, the comparison across 3 distinct age categories, and the precision in measurements of ocular biometry. A drawback was the lack of complete cycloplegic refraction in children in several studies, which jeopardized valid measurements of RE in this age category. Thus, we used AL/CR ratio as an indicator of RE to avoid heterogeneity in the outcome. This ratio has a high correlation with RE [Hashemi, et al. 2013; Ip, et al. 2007] and was available from all studies in the consortium. Another limitation was the lack of power to detect statistically significant differences between the age groups for most genes. A pooled analysis would have increased statistical power, but raw data from individual participants were not available. Ideally, a study using longitudinal data of the same children over different age periods would have the best study design for the current analysis.

Little has been reported on the development and progression of myopia as a function of age; however, a number of studies investigated the relationship between development of ocular biometry related to age. Until the age of 25 years, corneal curvature, the crystalline lens, and axial length all evolve with age, and thereby influence refractive error. The cornea increases in radius until preschool age leading to flattening of the corneal curvature and decrease in refractive power [Augusteyn, et al. 2012]; the crystalline lens grows until 10 years of age, also reducing refractive power [Mutti, et al. 2012; Mutti, et al. 1998]. This decrease in refractive power is compensated by axial elongation which increases from 17 mm in newborns [Lim, et al. 2015] to 23.3 mm in 12-13 year olds [French, et al. 2012]. The average AL in emmetropic adults is 23.5 mm [Fotedar, et al. 2010; Gordon and Donzis 1985]. The highest growth rate of AL occurs in the first years of life and relates to emmetropisation; the growth rate after early teens is more gradual but mainly relates to myopisation [Gordon and Donzis 1985]. The exact age at

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which eye growth stops is not known; generally this occurs before age 20 years, but increase in AL has been described up to the age of 25 years in university students [Fledelius 2000; Midelfart, et al. 1992].

One of the key detected GWAS-identified loci for refractive error is on chromosome 15 near the *GJD2* gene, that encodes a gap junction protein known as CX36. This protein not only processes cone-to-cone and cone-to-rod signals [Lee, et al. 2003] but also directs signaling between other retinal cells [Feigenspan, et al. 2001; Hidaka, et al. 2004]. This cell-to-cell communication appears to be under regulation of light exposure and dopamine [Bloomfield and Volgyi 2009], two factors that have an established role in eye growth and myopia development. Our data suggest that *GJD2* has an early-onset, indicating that altered retinal cell signaling, perhaps via reduced light exposure and low dopamine levels, may be a first step in myopia development. As expected, some early-onset genes also had a reported role in eye development. Knockout of *LAMA2,* a gene encoding the large extracellular glycoprotein laminin-α2; causes growth retardation including smaller eyes with compressed cellular layers [Gupta, et al. 2012]. Mutations in the serine protease gene *PRSS56* cause a severe decrease of AL leading to microphthalmia [Nair, et al. 2011]. Another developmental gene is *ZIC2,* an enhancer-binding factor required for embryonic stem cell specification [Luo, et al. 2015]. This gene may be important for development of retinal architecture, as it is known to be involved in differentiation and proliferation of retinal progenitor cells [Watabe, et al. 2011], and development of retinal ganglion cell trajectories [Herrera, et al. 2003]. Strikingly, several other genes involved in eye development, such as *SIX6, CDH7*, and *DLX1*, did not show an early onset but were more significant after the age of 10 years. Other early-onset genes were ion channels such as *KCNQ5*, a potassium channel present in cone and rod photoreceptors [Zhang, et al. 2011], and *CACNA1D*, a calcium channel present in photoreceptors [Xiao, et al. 2007]. *CHRNG* has as yet an unknown role in myopia development. It encodes the γ subunit of the embryonal

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acetylcholine receptor, which is widely expressed in the retina [Hruska, et al. 1978; Hutchins and Hollyfield 1985], and is associated with multiple pterygium syndrome [Vogt, et al. 2012].

Several remarkable patterns of effect were notable. For instance, the lead SNPs at the *BMP2, MYO1D, PTPRR*, and *BMP4* loci showed an opposite effect in children <10 years than in those who were older. This is not uncommon in biology, as such a trajectory has also been described for the *FTO* locus in relation to body mass index in children [Sovio, et al. 2011]. Interestingly, gene expression studies of *BMP2* in chickens showed that mRNA of this gene in the retinal pigment epithelium is up- or down-regulated depending on the location of the image plane [Zhang, et al. 2012]. When the image was focused behind the retina, mRNA was downregulated and the vitreous chamber enlarged. This underscores a bidirectional role for *BMP2* in modulation of eye growth.

Most genes had a late onset. *BMP4* has a similar function to *BMP2* as it is also responds to optical defocus with bidirectional regulation of eye growth [Zhang, et al. 2013]. *SIX6* is a DNA-binding homeobox and has a SIX domain, which binds downstream effector molecules. It is known to influence eye size in zebrafish with knocked down *SIX6* expression [Iglesias, et al. 2014]. Other genes play a less obvious role in myopiagenesis. MYO1D is involved in membrane trafficking in the recycling pathway and expressed in oligodendrites [Benesh, et al. 2012]. *RORB,* a gene encoding a nuclear receptor-directing photoreceptor differentiation, is known to activate and generate S-opsin [Jia, et al. 2009; Srinivas, et al. 2006]. DLX1 belongs to the DLX family of homeobox transcription factors, and produces GABAergic interneurons during embryonic development.

In conclusion, our study suggests that only a small proportion of the currently known GWAS-identified loci for RE exert their full effect at a young age. Furthermore, some of the pathways previously-identified by GWAS meta-analyses [Verhoeven, et al. 2013] can now be separated into early- and late-onset pathways. For example, genes coding for ion channels typically had an early onset, while genes related to connective tissue and visual feedback

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mechanisms appeared to become more important at a later age. As the currently known genes play only a minor role in early-onset myopia, we question whether this type of myopia is caused by common variants in other genes, or whether rare variants with large effects determine early-onset. Future research may shed more light on genes for early-onset myopia, and unravelling these genes will open up strategies for prevention of high myopia.

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Table I Participating studies and characteristics stratified per age group

Study	N	AL/CR (SD; range)	Age (SD)	Gender, % Female
Age <10 years				
STARS	207	2.99 (0.150; 2.76 – 3.46)	5.45 (2.11)	47.3
Generation R	3,874	2.87 (0.083; 2.38 – 3.90)	6.18 (0.51)	50.3
SCORM	898	3.02 (0.112; 2.63 – 3.45)	7.48 (0.87)	47.7
TEST	166	2.94 (0.101; 2.65 – 3.25)	7.53 (1.21)	52.4
GTES	345	2.97 (0.100; 2.62 – 3.45)	8.73 (0.79)	50.1
Total	**5,490**			
Age 10-25 years				
STARS	96	3.23 (0.127; 2.95 – 3.60)	12.23 (1.7)	58.3
GTES	699	3.13 (0.147; 2.58 – 3.82)	14.83 (1.2)	52.9
TEST	182	2.99 (0.108; 2.68 – 3.51)	15.16 (4.0)	60.4
ALSPAC	1,996	2.99 (0.099; 2.57 – 3.52)	15.46 (0.3)	53.6
BATS	983	3.03 (0.106; 2.67 – 3.82)	19.07 (3.2)	53,8
RAINE	1,044	3.05 (0.104; 2.63 – 3.54)	20.04 (0.4)	48.9
Total	**5,000**			
Age >25 years				
Nagahama	2,762	3.13 (0.153; 2.62 – 3.86)	52.05 (13.8)	49.0
Croatia-Split	730	3.02 (0.128; 2.38 – 3.90)	52.16 (13.0)	61.2
Croatia Korcula	832	2.99 (0.203; 2.26 – 5.73)	56.62 (13.3)	64.7
Croatia-Vis	573	2.99 (0.121; 2.50 – 3.83)	55.93 (13.8)	60.4
GHS 2	936	3.07 (0.160; 2.50 – 4.01)	59.26 (10.6)	50.0
GHS 1	1,919	3.06 (0.151; 2.30 – 3.88)	60.17 (10.7)	47.1
EPIC-Norfolk	6,051	3.05 (0.146; 2.42 – 3.95)	68.9 (8.0)	54.3
RS I-III	2,471	3.05 (0.143; 2.43 – 3.86)	70.02 (8.8)	53.6
Total	**16,274**			

*GTES= Guangzhou Twin Eye Study, RS I-III = Rotterdam Study I-III, GHS=Gutenberg Health Study

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Table II Effect size of myopia related genes in age groups <10 years, 10-25 years, 25> years

				<10 years		10 - 25 years		Combined		>25 years	
Variant	**Chr**	**Gene**	**RA**	**Beta (SE)**	**P**	**Beta (SE)**	**P**	**Beta (SE)**	**P**	**Beta (SE)**	**P**
Allele Score	-	-	-	**0.0019 (0.0003)**	**10^-11**	**0.0033 (0.0004)**	**10^-15**	**0.0024 (0.0002)**	**10^-24**	**0.0051(0.0003)**	**10^-72**
rs1652333	1	CD55	G	0.0033 (0.0017)	0.05	0.0006 (0.0024)	0.80	0.0026 (0.0014)	0.07	**0.0084(0.0017)**	**10^-6**
rs4373767	1	ZC3H11B	T	0.0010 (0.0017)	0.55	0.0032 (0.0023)	0.16	0.0019 (0.0014)	0.16	**0.0053(0.0017)**	**0.002**
rs17412774	2	PABPCP2	A	0.0007 (0.0017)	0.69	0.0010 (0.0023)	0.67	0.0008 (0.0014)	0.57	**0.0063(0.0017)**	**10^-4**
rs17428076	2	DLX1	C	0.0017 (0.0021)	0.43	0.0029 (0.0027)	*0.28*	0.0024 (0.0017)	0.16	**0.0073(0.0021)**	**10^-4**
rs1898585	2	PDE11A	T	0.0022 (0.0019)	0.26	0.0050 (0.0029)	0.09	**0.0034 (0.0017)**	**0.04**	**0.0057(0.0021)**	**0.007**
rs1656404	2	PRSS56	A	**0.0073 (0.0024)**	**0.002**	**0.0067 (0.0033)**	**0.04**	**0.0069 (0.0019)**	**10^-4**	**0.0079(0.0024)**	**0.001**
rs1881492	2	CHRNG	T	**0.0086 (0.0024)**	**10^-4**	0.0039 (0.0031)	0.21	**0.0064 (0.0020)**	**0.001**	**0.0085(0.0022)**	**10^-5**
rs14165	3	CACNA1D	G	0.0035 (0.0020)	0.08	**0.0082 (0.0026)**	**0.001**	**0.0055 (0.0016)**	**0.001**	**0.0055(0.0020)**	**0.005**
rs13091182	3	ZBTB38	G	0.0008 (0.0020)	0.69	-0.0001 (0.0024)	0.98	0.0007 (0.0015)	0.66	-0.0004(0.0019)	0.83
rs9307551	4	LOC100506035	A	0.0007 (0.0019)	0.70	0.0037 (0.0026)	0.16	0.0020 (0.0016)	0.20	**0.0051(0.0020)**	**0.008**
rs5022942	4	BMP3	A	0.0014 (0.0018)	0.44	-0.0016 (0.0026)	0.54	0.0007 (0.0015)	0.63	0.0006(0.0020)	0.78
rs7744813	6	KCNQ5	A	**0.0050 (0.0017)**	**0.004**	**0.0081 (0.0023)**	**10^-4**	**0.0060 (0.0014)**	**10^-5**	**0.0066(0.0018)**	**10^-4**
rs12205363	6	LAMA2	T	0.0041 (0.0041)	0.31	**0.0138 (0.0046)**	**0.003**	**0.0094 (0.0031)**	**0.003**	**0.0229(0.0036)**	**10^-10**
rs7829127	8	ZMAT4	A	0.0025 (0.0020)	0.22	0.0019 (0.0028)	0.49	0.0025 (0.0017)	0.13	**0.0072(0.0021)**	**0.001**
rs7837791	8	TOX	G	0.0029 (0.0016)	0.06	**0.0083 (0.0022)**	***10^-4***	**0.0050 (0.0013)**	**10^-4**	**0.0042(0.0017)**	**0.012**
rs4237036	8	CHD7	T	0.0001 (0.0018)	0.96	0.0032 (0.0024)	0.18	0.0013 (0.0014)	0.37	**0.0058(0.0018)**	**0.001**
rs11145465	9	TJP2	A	0.0035 (0.0022)	0.11	0.0027 (0.0028)	0.33	0.0029 (0.0017)	0.09	**0.0062(0.0021)**	**0.004**
rs7042950	9	RORB	G	0.0028 (0.0019)	0.14	0.0031 (0.0026)	0.24	0.0027 (0.0016)	0.08	**0.0071(0.0020)**	**10^-4**
rs7084402	10	BICC1	G	**0.0035 (0.0016)**	**0.03**	**0.0066 (0.0023)**	**0.004**	**0.0050 (0.0013)**	**10^-4**	**0.0074(0.0017)**	**10^-6**
rs6480859	10	KCNMA1	T	**0.0040 (0.0018)**	**0.02**	0.0037 (0.0023)	0.10	**0.0040 (0.0014)**	**0.004**	0.0015(0.0017)	0.38
rs745480	10	RGR	G	0.0007 (0.0016)	0.67	0.0021 (0.0022)	0.34	0.0011 (0.0013)	0.40	**0.0055(0.0017)**	**0.001**
rs10882165	10	CYP26A1	T	0.0012 (0.0018)	0.49	0.0002 (0.0024)	0.93	0.0007 (0.0014)	0.61	0.0011(0.0018)	0.54
rs1381566	11	LRRC4C	G	0.0026 (0.0020)	0.21	0.0040 (0.0034)	0.23	0.0028 (0.0018)	0.12	**0.0093(0.0022)**	**10^-5**
rs2155413	11	DLG2	A	0.0022 (0.0017)	0.18	0.0027 (0.0022)	0.23	0.0023 (0.0013)	0.09	0.0021(0.0017)	0.21
rs11601239	11	GRIA4	C	0.0011 (0.0016)	0.50	0.0027 (0.0022)	0.22	0.0014 (0.0013)	0.30	**0.0055(0.0017)**	**0.001**
rs3138144	12	RDH5	G	0.0020 (0.0021)	0.35	0.0039 (0.0027)	0.16	0.0028 (0.0017)	0.10	**0.0045(0.0019)**	**0.02**
rs12229663	12	PTPRR	A	-0.0023 (0.0019)	0.21	0.0046 (0.0026)	0.08	0.0000 (0.0016)	1.00	**0.0069(0.0019)**	**10^-4**
rs8000973	13	ZIC2	C	**0.0058 (0.0017)**	**10^-4**	**0.0058 (0.0023)**	**0.01**	**0.0059 (0.0014)**	**10^-5**	0.0027(0.0017)	0.10
rs2184971	13	PCCA	A	0.0008 (0.0016)	0.61	0.0006 (0.0023)	0.80	0.0009 (0.0014)	0.48	0.0021(0.0017)	0.21
rs66913363	14	BMP4	G	-0.0025 (0.0017)	0.15	0.0040 (0.0024)	0.10	0.0006 (0.0014)	0.68	**0.0047(0.0017)**	**0.006**
rs1254319	14	SIX6	A	0.0007 (0.0017)	0.68	0.0044 (0.0024)	0.07	0.0017 (0.0014)	0.22	**0.0054(0.0018)**	**0.002**
rs524952	15	GJD2	A	**0.0069 (0.0016)**	**10^-5**	**0.0068 (0.0023)**	**0.003**	**0.0067 (0.0013)**	**10^-7**	**0.0122(0.0016)**	**10^-14**
rs4778879	15	RASGRF1	G	0.0018 (0.0017)	0.29	0.0033 (0.0023)	0.15	0.0019 (0.0014)	0.17	**0.0051(0.0017)**	**0.002**
rs17648524	16	A2BP1	C	0.0018 (0.0018)	0.33	**0.0079 (0.0024)**	**0.001**	**0.0039 (0.0015)**	**0.01**	**0.0077(0.0019)**	**10^-5**
rs2969180	17	SHISA6	A	**0.0035 (0.0016)**	**0.03**	0.0017 (0.0023)	0.46	**0.0027 (0.0014)**	**0.05**	**0.0079(0.0017)**	**10^-6**
rs17183295	17	MYO1D	T	-0.0033 (0.0023)	0.16	0.0009 (0.0030)	0.76	-0.0018 (0.0018)	0.33	**0.0089(0.0023)**	**10^-4**

rs4793501	17	KCNJ2	T	0.0029 (0.0016)	0.08	0.0001 (0.0022)	0.95	0.0019 (0.0013)	0.16	**0.0041(0.0017)**	**0.015**
rs12971120	18	CNDP2	A	0.0002 (0.0019)	0.93	0.0048 (0.0026)	0.07	0.0017 (0.0015)	0.27	0.0024(0.0019)	0.22
rs235770	20	BMP2	T	**-0.0043 (0.0018)**	**0.02**	**0.0121 (0.0025)**	**10^-6**	0.0008 (0.0015)	0.60	**0.0043(0.0017)**	**0.013**

Values are betas (SE) and *P*-values, from linear regression models adjusted for sex, age and principal components if applicable meta-analysed with inversed variance meta-analysis in METAL. Bold: *P* <0.05.

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Figure I. Association between genetic risk score and myopia in the three age groups

Figure II. Association between non-weighted genetic risk score and AL/CR ratio in children and adults.

Figure III. Increased effect on AL/CR ratio with age for *BMP2* gene.

Figure IV. Distribution of effects on AL/CR ratio per myopia-related gene in three age groups

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Response to Reviewers' Comments

We thank the editor and reviewers for giving us the opportunity to revise our manuscript and the nice words. We provide a point-by-point response to the comments below. Underlined text represents changes according to the previous manuscript.

Comments to Author from reviewer:
Title: When do myopia genes have their effect? Comparison of genetic risks between children and adults
Authors: Tideman et al.
In this manuscript, authors investigated role of effects of GWAS identified SNPs on ocular biometry as a function of age. The investigators stratified the data into three groups: less than 10 years, 10-25 years, and over 25 years. The numbers of participants in each group were substantially large leading to good statistical power for associations studied in the paper. Overall, the paper is well-written. Specific comments:

1. It is not clear how the age group stratification decided? What is the rationale for the cut-offs of 10 and 25 years? Was this decided prior to analyses or post-hoc?

The group stratification was decided beforehand based on knowledge of myopia development and eye growth. Myopia can progress until 25 years; therefore, we chose this age cut off as the inclusion criteria for the adult GWAS study.[Verhoeven, et al. 2013] Mutti et al have shown that eye growth is larger in 6-9 years (0.69mm in 3 yrs) than in 10-13 years (0.27 mm in 3 years). Therefore, we chose 10 years as a cutoff in the children.[Zadnik, et al. 2003]

We added this to the methods: "*Age cut off points were based on prior knowledge regarding eye growth. The eye has the highest growth rate before the age of 10 years, and generally does not grow in axial length after age 25 years"*

2. Along the same lines, did you test for interaction between age and GRS (both as continuous variable)?

We indeed have tested for interaction between these variables, however, we did not obtain significant results (p-value 0.44). We think this is due to the narrow age range in the largest studies. The SD of age in Generation R is 0.5 years and in ALSPAC 0.3 years, which is too small to result in substantial differences in AL/CR ratio. The other studies are smaller and do not have enough power to obtain significant interaction terms.

3. Can you expand the results of the "Pathway analysis", page 7 and 8. It is not clear what the results are based on what is written.

Thank you for pointing this out. We have expanded this part in the result section page 8 in lines 15 – 50.

Verhoeven VJ, Hysi PG, Wojciechowski R, Fan Q, Guggenheim JA, Hohn R, MacGregor S, Hewitt AW, Nag A, Cheng CY and others. 2013. Genome-wide meta-analyses of multiancestry cohorts identify multiple new susceptibility loci for refractive error and myopia. Nat Genet 45(3):314-8.
Zadnik K, Manny RE, Yu JA, Mitchell GL, Cotter SA, Quiralte JC, Shipp M, Friedman NE, Kleinstein R, Walker TW and others. 2003. Ocular component data in schoolchildren as a function of age and gender. Optom Vis Sci 80(3):226-36.

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